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IMMUNOLOGICAL STUDIES IN MICE FOLLOWING IN UTERO EXPOSURE TO NiCl_2

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SUMMARY

The effect that NiCl_2 has on the development of immune function in mice was examined in the offspring of dams implanted with mini-osmotic pumps during pregnancy. Time bred C57BL/6J mice were implanted subcutaneously on day 5 of gestation with mini pumps which delivered a total dose of from 9.1 to 73.2 $\mu\text{g/g}$ NiCl_2 . The pumps delivered NiCl_2 to the dams through day 19 of gestation. At 8-10 weeks of age the offspring of NiCl_2 -dosed dams were evaluated for immune function. No consistent significant alterations were observed between control and treated offspring for the following: lymphoid organ or body weights; the lymphoproliferative response to B or T lymphocyte mitogens; the lymphoproliferative response to allogeneic spleen cells in the mixed lymphocyte reaction; the development of syngeneic tumors; or the primary antibody response to sheep red blood cells. Natural killer (NK) cell activity was reduced in offspring exposed to NiCl_2 in utero; however, the biological relevance of these reductions is questionable because of the failure to demonstrate an increased susceptibility to the B16-F10 syngeneic tumor. The results indicate that under the conditions and doses employed it appears that NiCl_2 does not adversely affect the developing immune system of the mouse.

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Abbreviations: Con A, concanavalin A; LP, lymphoproliferative; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; NK, natural killer; PHA, phytohemagglutinin; SRBC, sheep red blood cells.

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INTRODUCTION

We recently reported that single or multiple intramuscular injections of NiCl₂ in adult mice caused a significant reduction in a variety of T lymphocyte and natural killer (NK) cell mediated immune functions [1,2]. Suppression of the lymphoproliferative (LP) responses to concanavalin A (Con A) and phytohemagglutinin (PHA) along with a reduction in the number of theta positive T lymphocytes were observed in the spleens of NiCl₂ injected mice. Also, reductions in the primary antibody response to the T lymphocyte dependent antigen sheep red blood cells (SRBC), but not the T-independent antigen polyvinylpyrrolidone were observed following a single injection of NiCl₂ [1]. Suppressed NK cell activity of NiCl₂ injected mice was manifested as a reduction in the in vitro ⁵¹Cr release and the in vivo tumor clearance assays and as an enhancement in the development of tumor foci in the lungs of mice injected with B16-F10 melanoma cells [2].

Recently, there has been an increased interest in examining the immunotoxic effects of a variety of compounds administered during perinatal development (for reviews, see refs. [3,4]). Studies of immune ontogenesis have revealed that newborn mammals do not possess fully competent immune defense systems [5]. The development of the immune system in mammals involves a precise sequence of steps which begins early in gestation. Perturbation or abrogation of this developmental sequence of events can lead to immune dysfunctions which may be life-threatening [6]. It has been reported that the most profound effects of chemicals that modulate the immune system of animals occur when dosing of the compound begins during the development of the lymphoid system [7].

In light of the above, along with the fact that Ni compounds cross the placenta and can induce embryo and fetal toxicity [8-11], we decided to determine if exposure to Ni during in utero development might affect immune function measured during early adult life. We report here that NiCl₂ administered in pregnant mice via subcutaneously implanted mini-osmotic pumps does not adversely affect immune function in their offspring. With the possible exception of NK activity, no consistent significant effects were observed for a variety of immune function parameters which included the following: lymphatic organ and body weights; LP responses; and primary antibody response to SRBC.

MATERIALS AND METHODS

Animals and treatment

Male and female C57BL/6J mice, 10-12 weeks old (Jackson Lab., Bar Harbor, ME) were housed in plastic cages on wood shavings and provided food (Wayne Lab. Blox, Peoria, IL) and water ad libitum. Mice were held for at least 2 weeks following shipment prior to mating. Two female

mice were placed with 1 male and the following morning the females were checked for vaginal plugs. Plug positive females were randomly assigned to one of 3 or 4 groups. On day 5 after breeding, the female mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal® Sodium, Abbott Labs., Chicago, IL). Mice were weighed and, based on the average body weight, various concentrations of sterile NiCl_2 in saline were prepared from a stock solution (analyzed by neutron-activation, Dr. J.N. Weaver, North Carolina State University, Raleigh, NC). Mini-osmotic pumps (Model 2002, Alzet®, Alza Corp., Palo Alto, CA) which deliver at a rate of 0.5 $\mu\text{l/h}$ over a 14-day period, were filled with NiCl_2 solutions at final total doses of 0 (saline), 9.15, 18.3, 36.6 or 73.2 $\mu\text{g/g}$ NiCl_2 . The mini-pumps were surgically implanted subcutaneously midway on the dorsal area and secured using wound clips.

Mice implanted with mini-pumps were housed individually. At parturition the number of pups per litter was recorded. At weaning (21 days of age) male and female pups were separated and housed with pups of the same sex from other litters (one pup from each litter) within a treatment group.

Immune function assays

At 8–10 weeks of age, male and female offspring of NiCl_2 treated dams were evaluated for immune function. Non-littermates were used for immune function tests whenever possible with a maximum of 2 littermates/sex when insufficient numbers/group were available. Mice were killed by cervical dislocation and their body, spleen and thymus weights determined. Spleens were handled aseptically and cell suspensions were prepared for assay of LP as measured by [^3H]thymidine incorporation into DNA and NK cell activity as measured by ^{51}Cr release [1]. Briefly, spleen cells were suspended in RPMI-1640 medium, containing 25 mM Hepes, 2 mM L-glutamine (Grand Island Biological Co., Grand Island, NY), 50 $\mu\text{g/ml}$ gentamycin (Shering Corp., Union, NJ) and 5% heat-inactivated human type AB serum (Irvine Scientific, Irvine, CA). The 72-h LP response to mitogens of spleen cells from individual control and treated offspring was determined at optimal stimulating concentrations of the following: purified phytohemagglutinin (PHA, 0.2 $\mu\text{g/well}$, Burroughs Wellcome, Co., Research Triangle Park, NC); concanavalin A (Con A, 0.1 $\mu\text{g/well}$, Difco Lab., Detroit, MI); and *Escherichia coli* 0.28:B12 lipopolysaccharide (LPS, 2 $\mu\text{g/well}$, Difco). The one-way mixed lymphocyte reaction (MLR) of spleen cells was determined using mitomycin-C (Sigma, St. Louis, MO) treated allogeneic spleen cells from CBA/J mice (Jackson Lab.) as stimulator cells in a 96-h assay [12]. NK cell activity of spleen cell suspensions was determined using a 4 h *in vitro* ^{51}Cr release assay with YAC-1 mouse lymphoma cells as targets [2].

The development of tumor foci in the lungs of other offspring from control or Ni-treated dams was determined following the injection of B16-F10 melanoma cells [2]. B16-F10 cells (obtained from Dr. I. Fidler, National Cancer Institute, Frederick, MD), which were free of mycoplasma, were injected intravenously in unanesthetized mice at a concentration of 1×10^5

cells/mouse. Two weeks later mice were killed, their lungs removed and placed in Bouin's solution. Pulmonary tumor colonies were counted with the aid of a magnifying glass.

The primary antibody response to SRBC was determined in another group of offspring using the Cunningham modification of the Jerne plaque assay [13]. Mice were immunized with an intravenous injection of 0.2 ml of 5% SRBC. Four days later spleens were removed and the cells producing IgM anti-SRBC antibody were enumerated.

Statistical analysis

The data, excluding the lung tumor data, were analyzed by two-way analysis of variance with sex, dose, and dose by sex interaction effects examined using the General Linear Models Procedure of the Statistical Analysis System [14]. The purpose of this analysis was to determine whether or not the primary analysis should be done on males and females combined or separately. Williams' test [15] to determine the lowest dose at which a significant ($P < 0.01$) effect occurred was used as the primary analysis. The non-parametric Mann-Whitney U-test was used to analyze the B16-F10 lung tumor data.

RESULTS

Two separate experiments were performed in which mice were exposed to NiCl_2 in utero. Table I shows the birth records of mice in these 2 experiments. A decrease in litter size was observed with increasing concentration of

TABLE I

BIRTH RECORD OF MICE EXPOSED IN UTERO TO NiCl_2

Experiment	Dose ($\mu\text{g/g}$)	No. plug positive females	No. pregnant females ^a	No. litters ^b	$\bar{x}$ No. pups/ litter $\pm$ S.E. ^c
1	0	14	8	7	7.0 $\pm$ 0.4 (5-8)
	9.1	14	6	4	7.0 $\pm$ 0.7 (5-8)
	18.3	13	7	7	6.3 $\pm$ 0.7 (4-9)
	36.6	13	11	10	5.4 $\pm$ 0.7 (3-9) ^d
2	0	16	10	10	5.8 $\pm$ 0.8 (3-9)
	36.6	16	9	9	5.3 $\pm$ 0.5 (3-7)
	73.2	16	6	6	4.5 $\pm$ 0.7 (3-7)

^aNumber of females that delivered pups.

^bNumber of litters 2-3 days after birth. Loss of litters in Experiment 1 due to cannibalization of pups by the dam.

^cMean number of pups/litter, numbers in parentheses are range of pups/litter.

^d $P < 0.05$, Williams test.

NiCl₂ in both experiments. However, use of William's test showed that a significant ($P < 0.05$) difference in litter size occurred only at a dose of 36.6 $\mu\text{g/g}$ in the first experiment while no significant difference occurred at this or a higher dose (73.2 $\mu\text{g/g}$) in the second experiment. Litters in the first experiment were larger than those in the second experiment. The reason for the different litter sizes between these 2 experiments is not known. No gross malformations were observed in any of the control or treated litters.

In none of the preliminary analyses was there a significant ($P < 0.01$) dose by sex interaction. Therefore, the data for male and female offspring were combined within a dose group for the primary analysis. Examination of lymphoid organ and body weights of 8-week-old mice born to dams treated with NiCl₂ through pregnancy revealed no consistent significant changes (Table II). Body weight was significantly ($P < 0.01$) greater than controls at the highest dose in experiment 1 but was significantly decreased at a dose of 73.2 $\mu\text{g/g}$ in experiment 2. Thymus weights in offspring from both experiments were decreased at the highest dose of NiCl₂. While these decreases were not statistically significant, a significant ($P < 0.01$) decrease was observed in the thymus to body weight ratio of mice exposed to 36.6 $\mu\text{g/g}$ NiCl₂ in experiment 1.

Inconsistent changes were observed in the immune function of mice exposed to NiCl₂ in utero. Table III shows the LP response of spleen cells to mitogens and allogeneic cells, as measured by [³H]thymidine incorporation into DNA. In experiment 1 a significant ($P < 0.01$) decrease in [³H]thymidine incorporation was observed in non-stimulated cells from the highest dose group. This decrease, however, was not observed in the second experiment. Significant ($P < 0.01$) suppression of the LP response to the B lymphocyte mitogen LPS was observed for all doses of NiCl₂ in experiment 1. However, no change in this response was observed in experiment 2. No significant changes were observed in the LP response to the T cell mitogens Con A or PHA or to allogeneic cells in the MLR in either experiment.

Table III also shows NK activity, as measured by a 4-h ⁵¹Cr release assay, of mice exposed in utero to NiCl₂. A significant ($P < 0.01$) decrease in NK activity was observed in mice exposed to both doses of NiCl₂ in experiment 2. In experiment 1, while there was a decrease in NK activity with increasing dose of NiCl₂, no significant differences were observed. It is important to point out that while a significant difference was observed in experiment 2 at an effector to target (E:T) cell ratio of 100:1, no significant differences were observed at E:T ratios of 25:1 or 50:1 (data not shown).

IgM PFC/10⁶ spleen cells were significantly enhanced in experiment 1 at 18.3 and 36.6 $\mu\text{g/g}$ NiCl₂ but not in experiment 2 at 36.6 $\mu\text{g/g}$ (Table IV). No significant enhancement of PFC/spleen was observed in either experiment, although an increase (albeit not significant) was observed in experiment 1.

Table IV also shows the results of an experiment in which the development of tumor foci in the lungs was examined in mice injected with B16-F10 melanoma cells. No significant change in the development of lung tumors was observed for control or Ni-treated mice.

TABLE II
THE EFFECT OF IN UTERO EXPOSURE TO NiCl_2 ON MOUSE LYMPHOID ORGAN AND BODY WEIGHT

Experiment ^a	Dose ^b ($\mu\text{g/g}$)	Body wt (g)	Spleen wt (mg)	Thymus wt (mg)	Spleen wt		Thymus wt	
					Body wt ($\times 10^{-3}$)		Body wt ($\times 10^{-3}$)	
1	0 (14) ^c	23.1 $\pm$ 1.1	80.5 $\pm$ 2.5	54.7 $\pm$ 2.7	3.55 $\pm$ 0.15		2.48 $\pm$ 0.21	
	9.1 (8)	24.6 $\pm$ 1.3	90.6 $\pm$ 1.4	53.5 $\pm$ 5.3	3.75 $\pm$ 0.19		2.27 $\pm$ 0.31	
	18.3 (14)	24.2 $\pm$ 0.9	87.1 $\pm$ 5.3	55.0 $\pm$ 3.1	3.62 $\pm$ 0.21		2.34 $\pm$ 0.19	
	36.6 (19)	25.0 $\pm$ 0.9 ^d	85.9 $\pm$ 2.4	47.0 $\pm$ 2.7	3.54 $\pm$ 0.18		1.96 $\pm$ 0.16 ^d	
2	0 (16)	24.4 $\pm$ 1.0	88.1 $\pm$ 1.6	68.3 $\pm$ 2.7	3.72 $\pm$ 0.19		2.92 $\pm$ 0.21	
	36.6 (15)	25.5 $\pm$ 1.1	85.9 $\pm$ 1.7	63.7 $\pm$ 2.6	3.49 $\pm$ 0.21		2.59 $\pm$ 0.19	
	73.2 (16)	23.1 $\pm$ 0.9 ^d	84.0 $\pm$ 1.5	61.3 $\pm$ 3.9	3.73 $\pm$ 0.18		2.76 $\pm$ 0.24	

^aMice were 8 weeks old at time of assay.

^bTotal dose to pregnant dam over 14 days.

^cNumber of offspring assayed.

^d $P < 0.01$, Williams' test.

TABLE III

LYMPHOPROLIFERATIVE RESPONSES TO MITOGENS AND ALLOGENEIC CELLS, AND NATURAL KILLER CELL ACTIVITY OF SPLEEN CELLS FROM MICE EXPOSED TO NiCl_2 IN UTERO

Experiment ^a	Dose ^b ($\mu\text{g/g}$)	Mean CPM ($\times 10^{-1}$) $\pm$ S.E.		Mean % ^{51}Cr Release $\pm$ S.E.			
		Cells	Con A	PHA	LPS	MLR	NK Activity ^c
1	0 (14) ^d	0.13 $\pm$ 0.02	27.6 $\pm$ 2.67	26.5 $\pm$ 1.27	17.2 $\pm$ 1.14	8.8 $\pm$ 0.83	20.2 $\pm$ 1.07
	9.1 (8)	0.11 $\pm$ 0.01	33.4 $\pm$ 2.05	32.9 $\pm$ 2.09	13.6 $\pm$ 0.56 ^e	8.8 $\pm$ 0.84	19.6 $\pm$ 1.36
	18.3 (14)	0.10 $\pm$ 0.01	22.6 $\pm$ 1.74	25.8 $\pm$ 1.49	12.1 $\pm$ 0.55 ^e	6.2 $\pm$ 0.83	19.2 $\pm$ 0.82
	36.6 (19)	0.08 $\pm$ 0.01 ^e	20.6 $\pm$ 1.81	26.1 $\pm$ 0.94	12.9 $\pm$ 0.97 ^e	7.4 $\pm$ 0.83	18.1 $\pm$ 0.81
2	0 (16)	0.07 $\pm$ 0.01	30.1 $\pm$ 2.16	37.1 $\pm$ 1.21	9.7 $\pm$ 0.53	39.5 $\pm$ 2.34	17.4 $\pm$ 0.71
	36.6 (15)	0.09 $\pm$ 0.01	35.6 $\pm$ 2.02	39.3 $\pm$ 1.18	10.5 $\pm$ 0.51	37.0 $\pm$ 2.22	14.3 $\pm$ 1.02 ^e
	73.2 (16)	0.07 $\pm$ 0.01	33.5 $\pm$ 2.65	41.0 $\pm$ 1.49	9.7 $\pm$ 0.46	42.9 $\pm$ 2.84	14.1 $\pm$ 0.43 ^e

^aMice were 8 weeks old at time of assay.

^bTotal dose to pregnant dams over 14 days.

^c100:1 effector to target cell ratio.

^dNumber of offspring assayed.

^e $P < 0.01$, Williams' test.

TABLE IV

PRIMARY ANTIBODY RESPONSE TO SHEEP RED BLOOD CELLS AND DEVELOPMENT OF B16-F10 LUNG TUMOR FOCI IN MICE EXPOSED TO NiCl_2 IN UTERO

Experiment	Dose ($\mu\text{g/g}$)	Primary antibody response		Lung tumors ^b
		Mean PFC/10 ⁴ cells $\pm$ S.E.	Mean PFC ($\times 10^{-4}$)/ spleen $\pm$ S.E.	Mean tumors/ lung $\pm$ S.E.
1	0 (13) ^c	731 $\pm$ 82	0.80 $\pm$ 0.10	19.1 $\pm$ 2.6 (13)
	9.1 (5)	726 $\pm$ 73	0.96 $\pm$ 0.12	20.0 $\pm$ 5.4 (6)
	18.3 (9)	1097 $\pm$ 103 ^d	1.48 $\pm$ 0.11	19.4 $\pm$ 5.3 (11)
	36.6 (10)	1022 $\pm$ 81 ^d	1.33 $\pm$ 0.14	15.2 $\pm$ 2.5 (17)
2	0 (22)	602 $\pm$ 36	1.11 $\pm$ 0.06	ND ^e
	36.6 (14)	671 $\pm$ 58	1.19 $\pm$ 0.11	ND

^aMice were 9 weeks old at time of assay.^bMice were injected intravenously with B16-F10 melanoma cells at 8 weeks of age and tumors counted 2 weeks later. Number in parentheses is number of mice/dose injected with B16-F10 cells.^cNumber of offspring assayed.^d $P < 0.01$, Williams' test.^eNot determined.

DISCUSSION

Prenatal exposure to Ni has been reported to be embryotoxic, fetotoxic and/or teratogenic in rats [10,11,16], hamsters [17], chickens [18] and mice [19]. Studies in rats [10] and mice [8,19] indicate that NiCl_2 is able to cross the placenta and enter the fetuses. In the present study decreases in litter size were observed in both experiments (Table I), although a statistically significant reduction in litter size was observed only in experiment 1 at a dose of 36.6 $\mu\text{g/g}$ NiCl_2 . In the second experiment no statistically significant reduction in litter size was observed even at a higher dose of 73.2 $\mu\text{g/g}$. The reason for this discrepancy is not known but may be due to the overall smaller litters in experiment 2. There is no indication that exposure to NiCl_2 at the doses employed affected the pregnancy rate. Furthermore, no gross malformations were observed in any of the pups born to dams treated with NiCl_2 .

Despite the fact that in utero exposure of mice to NiCl_2 caused a reduction in litter size, no clear indication of a persistent change in the body weights of offspring was observed. One would expect that a reduction in litter size might result in litters with larger pups. This appears to be the case in experiment 1 in which offspring of dams dosed at 36.6 $\mu\text{g/g}$ NiCl_2 were significantly ($P < 0.01$) larger than controls at 8 weeks of age. This hypothesis; however, is not supported by the results of experiment 2 in which offspring exposed in utero to 73.2 $\mu\text{g/g}$ NiCl_2 had significantly ($P < 0.01$) reduced body weights. The reason for this discrepancy is not known.

A reduction in thymus weight, albeit non-significant, was observed in both experiments. The observed significant decrease in thymus to body weight ratio at the highest NiCl_2 dose in experiment 1 is not only a function of decreased thymus mass but also reflects the larger body weight in this group. These results are nonetheless interesting in that reduced thymus weight is observed in adult mice given a single intramuscular injection of NiCl_2 [1].

The LP response data show no consistent effect of in utero exposure to NiCl_2 at doses which caused effects in adults [1]. Such a discrepancy is not uncommon in immune function studies because of the great amount of variability in this assay. The reduction in [^3H]thymidine incorporation following stimulation with the B cell mitogen LPS in experiment 1 was not reproduced in experiment 2 (Table III). The results indicate that spleen cells from offspring of NiCl_2 exposed dams maintain their ability to respond to mitogens and to allogeneic spleen cells in the one-way mixed lymphocyte reaction. These results are contrary to earlier studies in which spleen cells from adult mice injected with NiCl_2 had suppressed responses to the T cell mitogens Con A and PHA but not the B cell mitogen LPS [1]. In this earlier work the decrease in T cell mitogen responsiveness was correlated with decreased numbers of theta positive T lymphocytes in the spleens of NiCl_2 treated mice [1].

The observed reduction in NK cell activity agrees with earlier work in adult mice [1,2]. Repeated injection of adult mice with NiCl_2 over a 2-week period, at total dosages of 18.3, 36.6 and 73.2 $\mu\text{g/g}$, caused significant reductions in NK activity [2]. In the present study reductions in NK activity in experiment 2 were different at 36.6 and 73.2 $\mu\text{g/g}$ NiCl_2 compared with controls. In the first experiment non-significant reductions were observed in NK activity for all the dose groups. While the NK activity data suggest an effect of in utero exposure to NiCl_2 , it must be pointed out that although reductions in NK activity were observed with increasing NiCl_2 dose in both experiments only the 100:1 E:T ratio data of experiment 2 were statistically ($P < 0.01$) different. Another important consideration is the biological relevance of these small reductions in NK activity. NK cells have been shown to be the effector cells responsible for in vitro and in vivo activity against a variety of tumors including B16-F10 melanoma cells [20,21]. In earlier work a 50% reduction in NK activity, by a single injection of 18.3 $\mu\text{g/g}$ NiCl_2 , was required to produce a significant enhancement in the development of B16-F10 tumor foci [2]. In the present study a dose of 36.6 $\mu\text{g/g}$, which caused a 10% and 18% reduction in NK activity in experiments 1 and 2, respectively, did not result in any change in the number of B16-F10 tumor foci in lungs (Table IV).

An inconsistent primary antibody response to SRBC was observed in offspring exposed to NiCl_2 in utero. In the first experiment a significant ($P < 0.01$) increase in PFC/ 10^6 cells was observed at 18.3 and 36.6 $\mu\text{g/g}$ NiCl_2 . An increase in the total PFC/spleen was also observed in experiment 1, albeit not significant. In experiment 2, however, mice exposed at a dose of 36.6 $\mu\text{g/g}$ showed no change in the PFC response. The data in experiment 1

are different from those reported for adult mice given a single injection of NiCl_2 in which a reduction in both the PFC/ 10^6 cells and PFC/spleen was observed [1].

The failure to demonstrate a consistent change in the primary response to SRBC and in the LP response suggests that in utero exposure to NiCl_2 does not affect these immune parameters 8–10 weeks after exposure. This conclusion is supported by the fact that although "statistically significant" differences were observed between control and treated offspring, one would not expect to find definitive correlations between immune function assays and host resistance assays when the former were not of sufficient magnitude to expect biological relevance.

Immune parameters were evaluated at 8–10 weeks of age because immune function in mice is fully mature at this time. Evaluation of immune function in younger mice is complicated by this and the fact that suppressor cells may play a significant role in regulating immune responses [22,23]. On the other hand it is possible that any effects that Ni has on the developing immune system may have been missed because of the time lag between exposure and function assessment. One would expect that if Ni adversely and irreversibly affected stem cells that a persistent change in immune function would be observed. This was not the case, however, which suggests that immune effects of Ni may be reversible, as observed in adults [1].

The NK data, while suggestive of a consistent decrease in activity with increasing dose of NiCl_2 , cannot be unequivocally ascribed to this in utero treatment. However, the results of this study in addition to those of earlier studies [1,2] provides further evidence for a causal relationship between Ni exposure and decreased NK cells activity. As further evidence for this, it has been reported that rats injected intramuscularly with Ni_3S_2 , which subsequently developed rhabdomyosarcomas, displayed a long lasting (up to 28 weeks) depression of NK activity [24]. In addition, we have found that exposure of adult rats to NiCl_2 as a single intramuscular injection causes a significant reduction in NK activity (unpublished results). Further research is warranted to elucidate the apparent association between Ni exposure and reduced NK activity.

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